Molecular Mechanism of Altered Ezetimibe Disposition in Nonalcoholic Steatohepatitis

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ABSTRACT:

Ezetimibe (EZE) lowers serum lipid levels by blocking cholesterol uptake in the intestine. Disposition of EZE and its pharmacologically active glucuronide metabolite (EZE-GLUC) to the intestine is dependent on hepatobiliary efflux. Previous studies suggested that hepatic transporter expression and function may be altered during nonalcoholic steatohepatitis (NASH). The purpose of the current study was to determine whether NASH-induced changes in the expression and function of hepatic transporters result in altered disposition of EZE and EZE-GLUC. Rats fed a methionine- and choline-deficient (MCD) diet for 8 weeks were administered 10 mg/kg EZE either by intravenous bolus or oral gavage. Plasma and bile samples were collected over 2 h followed by terminal urine and tissue collection. EZE and EZE-GLUC concentrations were determined by liquid chromatography-tandem mass spectrometry. The hepatic expression and function of uptake transporters (Fisher et al., 2009a) in rodent models of simple steatosis and NASH. These studies have led us to hypothesize that NASH, in particular, causes significant disruption in hepatic metabolism and disposition of administered pharmaceuticals due to alterations in hepatic drug transporters. However, the precise mechanisms underlying these dispositional alterations in rodents and their manifestation in human NAFLD remain elusive.

Ezetimibe (EZE) is an orally administered cholesterol absorption inhibitor that acts primarily via inhibition of Niemann-Pick C1-like 1 (NPC1L1) at the villus tip of enterocytes of the small intestine (Garcia-Calvo et al., 2005; Kosoglou et al., 2005). EZE and EZE-GLUC is then metabolized (80%) to a glucuronide metabolite (EZE-GLUC) (Garcia-Calvo et al., 2005; Kosoglou et al., 2005). EZE is quickly metabolized (~80%) to a glucuronide metabolite (EZE-GLUC) within enterocytes, followed by delivery of parent drug and metabolite, via the portal vein, to the liver where additional EZE glucuronidation occurs (Kosoglou et al., 2005). EZE and EZE-GLUC is then patients with NAFLD correlates with hepatic histological severity, with patients with NASH exhibiting numerous symptoms (Marchesini et al., 2003; McCullough, 2006; Fan, 2008). Because of the prevalence of metabolic syndrome in patients with NAFLD and the multiplicity of symptoms (dyslipidemia, hyperinsulinemia, central adiposity, and hypertension), patients with NAFLD may often be medicated for several symptoms. We have previously investigated the effect of NAFLD on the disposition of acetaminophen (Lickteig et al., 2007) and the function of uptake transporters (Fisher et al., 2009a) in rodent models of simple steatosis and NASH. This work was supported by the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases [Grant DK068039]; the National Institutes of Health National Institute of Environmental Health Sciences [Grant ES006694]; the National Institutes of Health National Center for Complementary and Alternative Medicine [Grant AT002842]; and the National Institutes of Health National Institute of Child Health and Human Development [Grant HD062489]. The Liver Tissue Cell Distribution System was sponsored by the National Institutes of Health [Contract N01-DK70004/HHSN267200700004C].

ABBREVIATIONS: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; EZE, ezetimibe; NPC1L1, Niemann-Pick C1-like 1; EZE-GLUC, ezetimibe glucuronide; ABC, ATP-binding cassette; MCD, methionine and choline deficient; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS/MS, tandem mass spectrometry; Mrd, multidrug resistance; Ugt, UDP glucuronosyltransferase; ERK, extracellular signal-regulated kinase.
excreted into bile and returned to the small intestinal lumen for inhibition of NPC1L1 (Kosoglou et al., 2005). Enterohepatic recycling of EZE and EZE-GLUC is thought to be an important determinant of the long half-life and efficacy of EZE because both parent and metabolite are pharmacologically active; however, EZE-GLUC is believed to be a more potent inhibitor of NPC1L1 and accounts for the vast majority of drug (80–90%) measured in biological compartments (Kosoglou et al., 2005).

Studies in knockout animals have demonstrated the importance of several efflux drug transporters in the disposition of EZE and EZE-GLUC. In particular, Abcc2 and Abcb1 have been implicated as having a major role in the biliary excretion of EZE-GLUC (Oswald et al., 2006b, 2007, 2010), whereas Abcc3 mediates sinusoidal efflux (de Waart et al., 2009). Under normal physiological conditions, EZE is eliminated primarily via feces in unconjugated form, which may be due to hydrolysis of the EZE-GLUC secreted into bile (Kosoglou et al., 2005). However, in the absence of Abcc2, EZE disposition into the blood from the liver increases and leads to an increase in urinary excretion of EZE and EZE-GLUC (Oswald et al., 2006b). Diminished biliary excretion of EZE-GLUC and concomitant increased urinary excretion may have an effect on drug half-life and overall systemic exposure, necessitating dosage adjustments. In the present study, we examined the effect of NASH on the disposition of EZE and its major glucuronide metabolite. EZE is administered orally to humans; however, the expression profile of drug transporters in the methionine- and choline-deficient (MCD) diet rodent model has not been characterized. We therefore chose to dose both orally and intravenously to discern the hepatic contribution to EZE disposition in NASH. In addition, we have conducted expression and localization analyses of major hepatic efflux drug transporters involved in the disposition of EZE and EZE-GLUC in the MCD diet rodent model of NASH to more clearly understand the potential effects of NAFLD on clinical drug disposition.

Materials and Methods

Materials. Ezetimibe (E975000) was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada) and was determined to be 99.8% pure. Urethane, carboxymethylcellulose, ethanol, propylene glycol, polyethylene glycol, HPLC-grade methanol, HPLC-grade methyl tert-butyl ether, and β-glucuronidase (5000 U) were obtained from Sigma-Aldrich (St. Louis, MO). Neutral-buffered formalin (10%) and HPLC-grade H2O were acquired through Thermo Fisher Scientific (Waltham, MA).

Animals. Male Sprague-Dawley rats weighing 200 to 250 g were obtained from Harlan (Indianapolis, IN). All animals were acclimated in 12-h light/dark cycles in a University of Arizona Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facility for at least 1 week before experiments and were allowed water and standard chow ad libitum. Housing and experimental procedures were in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute FIG. 1. Hepatic mRNA expression in diet-induced NASH: mRNA levels in rats fed either a control or MCD diet for 8 weeks. mRNA levels were measured by the branched DNA assay and expressed as relative lights units (RLU) per 10 μg of total RNA. Data are presented as means ± S.E.M. *, significant difference from control with a significance level of p ≤ 0.05.
of Laboratory Animal Resources, 1996). Rats were fed a control diet with methionine and choline resupplemented or a methionine-choline deficient (MCD) diet (Dyets Inc., Bethlehem, PA) for 8 weeks. At the conclusion of 8 weeks and before initiation of EZE experiments, control animals weighed an average of 400.006 ± 6.895 g and MCD/NASH animals weighed an average of 194.36 ± 2.283 g. Weight loss in the MCD model is a common and well documented side effect (Fan and Qiao, 2009; Schattenberg and Galle, 2010).

**Ezetimibe Disposition Experiments.** After 8 weeks of the respective diet treatment, animals were administered a bolus dose of urethane (1.2 g/kg i.p., w/v). The femoral artery and vein were cannulated with PE-50 polyethylene tubing (Braintree Scientific, Braintree, MA), and the common bile duct was cannulated with PE-10 polyethylene tubing (Braintree Scientific) distal to the bile duct bifurcation. Animal core temperature was maintained throughout collection of bile with a TCAT-2V temperature monitor and heat pad (Physitemp Instruments Inc., Clifton, NJ). Animals were administered a 10 mg/kg dose (5 ml/kg) of EZE either orally (n = 3–5, prepared in 0.25% carboxymethylcellulose) or intravenously through the femoral vein cannula (n = 3–5, prepared in 10% ethanol, 40% propylene glycol, 30% PEG 400, and 20% sterile H2O). Blood samples (100 μl) were drawn from the femoral artery cannula at 0, 2, 10, 20, 40, 60, 90, and 120 min. Bile samples were collected in chilled tubes at 15-min intervals for 120 min after EZE administration. Terminal urine samples were collected via bladder puncture and collected in preweighed tubes. All samples were stored at −80°C until use. After sample collections, animals were euthanized while still under anesthesia. After euthanasia, liver slices for histological analysis were collected and placed in 10% neutral-buffered formalin for 24 h followed by 70% ethanol until paraffin embedding was performed by the University of Arizona Histology Service Laboratory. The remaining liver tissue was snap-frozen in liquid nitrogen and stored at −80°C until further use. Small intestines were sectioned by forming a “Z” with the full length of the organ, differentiating the duodenum, jejunum, and ileum in order. Tissue samples for the duodenum and ileum were taken from the middle of the first and third arms of the Z, flushed with sterile H2O, snap-frozen in liquid nitrogen, and stored at −80°C until use.

**Sample Preparation.** All reagents used were of HPLC-grade quality. Sample were prepared according to the methods of Oswald et al. (2006a, 2007) with slight modifications for smaller volumes. For determination of EZE, 25 μl of plasma, urine, or bile was mixed with 2 μl of hydroxychalcone internal standard solution. The mixture was then diluted with 0.4 ml of H2O. Samples were then extracted with 0.8 ml of methyl tert-butyl ether for 15 min followed by centrifugation at 4000 rpm for 2 min. The organic layer was transferred to

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**Fig. 2.** Hepatic protein expression in diet-induced NASH: protein levels in rats fed either a control or MCD diet for 8 weeks. Immunoblots are shown with total ERK as the control protein. Relative protein levels were determined by densitometric analysis and expressed relative to total ERK. Data are presented as means ± S.E.M. * significant difference from control with a significance level of p ≤ 0.05.
a clean tube. The extraction was then repeated, and the resulting combined organic layers were evaporated under a gentle stream of N₂ at 50°C. The residue was then dissolved in 77.8% aqueous methanol. For determination of total EZE (parent plus conjugated EZE), before the above described protocol, 90 μl of H₂O and 10 μl of β-glucuronidase (>5000 U) were added to the 25-μl sample and incubated at 50°C for 60 min. After cooling, the above-described protocol was resumed with dilution of the sample in 0.3 ml of H₂O. For determination of total EZE (parent plus conjugated EZE), before the above described protocol, 90 μl of H₂O and 10 μl of β-glucuronidase (>5000 U) were added to the 25-μl sample and incubated at 50°C for 60 min. After cooling, the above-described protocol was resumed with dilution of the sample in 0.3 ml of H₂O. For determination of total EZE (parent plus conjugated EZE), before the above described protocol, 90 μl of H₂O and 10 μl of β-glucuronidase (>5000 U) were added to the 25-μl sample and incubated at 50°C for 60 min. After cooling, the above-described protocol was resumed with dilution of the sample in 0.3 ml of H₂O. For determination of EZE in liver tissue, 500 mg of tissue was homogenized in 2.5 ml of H₂O. Then, 0.1 ml of homogenate was mixed with 0.2 ml of H₂O and 2 μl of internal standard solution. The protocol was then continued as described above for plasma, urine, and bile. To determine total EZE in liver tissue, 0.1 ml of the prepared tissue homogenate was combined with 90 μl of H₂O and 10 μl of β-glucuronidase. The mixture was incubated for 60 min at 50°C. Then the sample was allowed to cool and diluted with 0.2 ml of H₂O and 2 μl of internal standard solution. Extractions were performed as described above.

**Determination of Total EZE, EZE, and EZE-GLUC Concentrations.** LC-MS/MS detection of total EZE, EZE, and EZE-GLUC was conducted on the basis of the method of Oswald et al. (2006a) in the Arizona Laboratory for Emerging Contaminants. The LC-MS/MS system was composed of a Waters-Micromass Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA) and an Acquity UPLC system with an auto-sampler (Waters) equipped with MassLynx 4.1 software (Waters). The chromatography was performed with a gradient beginning at 20:80 (v/v) acetonitrile-water and ending at 80:20 (v/v) acetonitrile-water for 7 min with a flow rate of 0.25 ml/min on an Acquity UPLC BH C18 (1.7 μm; 2.1 × 50 mm) column (Waters). The mass spectrometer was used in multiple reaction monitoring mode and equipped with an electrospray ionization source in the negative mode. The m/z transitions monitored were 408 to 271 for EZE and 223 to 117 for internal standard. The concentration of EZE was determined for all samples submitted. EZE-GLUC concentrations were calculated as the difference between total EZE and EZE measurements.

**RNA Preparations.** Total RNA was isolated from rodent liver and intestinal tissue using RNAzol B reagent (Tel-Test Inc., Friendswood, TX) per the manufacturer’s recommendations. RNA concentrations were determined by UV spectrophotometry, and the integrity of the RNA was confirmed by ethidium bromide staining after agarose gel electrophoresis.

**Protein Preparations.** Whole-cell lysate preparations of rodent liver and intestinal tissue were prepared from ~300 mg of tissue homogenized in NP-40 buffer [20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 2 mM EDTA with 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 25 ml] at 4°C. Homogenized tissue was then agitated at 4°C for 2 h and centrifuged at 10,000 g for 30 min, and the supernatant was transferred to a clean collection tube. Protein concentrations were determined using the Pierce BCA Protein Quantitation Assay (Thermo Fisher Scientific) per the manufacturer’s recommendations.

**Branched DNA Assay.** Specific oligonucleotide probes for Abcc2 and Abcc3, Mdr1a and Mdr1b (Brady et al., 2002; Cherrington et al., 2002), and Ugt1a1 (Vansell and Klaassen, 2002) were diluted in lysis buffer supplied by the Quantigene HV Signal Amplification Kit (Genospectra, Fremont, CA). Substrate solution, lysis buffer, capture hybridization buffer, amplifier, and label probe buffer used in the analysis were all obtained from the Quantigene.

![Fig. 3. Effect of diet-induced NASH on plasma EZE, EZE-GLUC, and total EZE concentrations. After 8 weeks of control (△) and MCD (●) diet feeding, EZE disposition experiments were conducted. Femoral artery and vein and bile duct cannulations were performed, and animals were administered either an oral or intravenous dose of 10 mg/kg EZE. Plasma samples were collected beginning 2 min after dose until 120 min. Concentrations of total EZE and EZE were determined by LC-MS/MS; EZE-GLUC concentrations were calculated as the difference between total EZE and EZE. Data are presented as means ± S.E.M. *, significant difference from control for each time point with a significance level of p ≤ 0.05.](dmd.aspetjournals.org)
Discovery Kit (Genospectra). The assay was performed in 96-well format with 10 µg (liver) or 5 µg (intestine) of total RNA added to the capture hybridization buffer and 50 µl of the diluted probe set. The total RNA was then allowed to hybridize to the probe set overnight at 53°C. Hybridization steps were performed per the manufacturer’s protocol on the following day. Luminescence of the samples was measured with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex Data Management Software (version 5.02).

Immunoblot Protein Analysis. Whole-cell lysate proteins (80 µg/well, liver and 40 µg/well, intestine) were separated by SDS-polyacrylamide gel electrophoresis on 10% gels and transferred to polyvinylidene difluoride membranes overnight. The following mouse monoclonal antibodies were obtained from Abcam, Inc. (Cambridge, MA) and used to determine relative protein levels: Abcc3 (MjII-9) and Abcb1 (C219). Abcc2 (MjIII-5) protein levels were determined using a mouse monoclonal antibody obtained from Kamiya Biomedical Company (Seattle, WA). Protein levels of Ugt1a1 were determined using a rabbit polyclonal antibody (Abcam, Inc.). Relative protein expression was quantitated using image processing and analysis with ImageJ software (National Institutes of Health, Bethesda, MD) and normalized to total ERK (C-16 and C-14; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Bile Acid Concentrations. Bile acids were measured in rodent bile samples at time 0 of disposition studies. Bile samples were diluted 1 µl in 49 µl of sterile-filtered saline. A Diazyme Total Bile Acids Assay Kit (Diazyme Laboratories, Poway, CA) was used to determine total bile acid concentrations spectrophotometrically over a 1-min interval at 405 nm per the manufacturer’s instructions. The assay was calibrated using a manufacturer-provided standard 50 µM bile acid calibrator solution.

Immunohistochemistry. Immunohistochemical staining for all proteins was performed on formalin-fixed, paraffin-embedded samples. In brief, tissue sections were deparaffinized in xylene and rehydrated in ethanol, followed by antigen retrieval in citrate buffer (pH 6.0, Abcb1) or Tris-EDTA buffer (pH 9.0, Abcc2). Endogenous peroxidase activity was blocked with 0.3% (v/v) H2O2 in methanol for 20 min. Immunohistochemical staining for Abcb1 was performed with a MACH3 staining kit (Biocare Medical, Concord, CA) per the manufacturer’s protocol. Samples were incubated in a primary antibody (antibodies described above) solution overnight at 4°C. Immunohistochemical staining for Abcc2 was performed with the MACH4 staining kit (Biocare Medical) per the manufacturer’s recommendations. Abcc2 antibody incubation was performed overnight at 4°C. All slides were imaged with a Nikon Eclipse E4000 microscope and a Sony Exwave DXC-390 camera.

Statistical Analysis. For disposition studies, a Student’s t test was used to determine significant differences between diet groups at all time points. All subsequent data were analyzed by Student t test to determine significant differences between diet groups. All analyses were performed with Stata10 software (StataCorp LP, College Station, TX), and a significance level of p ≤ 0.05 was used for all data analyses.

Results

Hepatic Gene and Protein Expression in Diet-Induced NASH. mRNA levels of Abcc2, Abcc3, Abcb1a, Abcb1b, and Ugt1a1 were determined by the branched DNA method of RNA quantification in control and MCD rat livers, and the results are shown in Fig. 1. In

![Oral Dosing](image1)

![Intravenous Dosing](image2)

Fig. 4. Effect of diet-induced NASH on biliary concentrations of EZE, EZE-GLUC, and total EZE. The experimental and analytical conditions were the same as those described for Fig. 3. Bile concentrations of EZE, EZE-GLUC, and total EZE are shown in control (△) and MCD (□) rodents. After EZE dosing, bile was collected at 15-min intervals over a 120-min period. Data are presented as means ± S.E.M. * significant difference from control for each time point with a significance level of p ≤ 0.05.
MCD livers, Abcc3 mRNA levels were significantly increased (44.8-fold) from control. In addition, Abcb1a and Abcb1b mRNA levels were both significantly elevated in MCD livers (3.8- and 17.3-fold, respectively). Abcc2 and Ugt1a1 were not altered at the transcriptional level.

Figure 2 shows relative protein levels of Abcc2, 3, Abcb1, and Ugt1a1 in control and MCD rat livers as determined by immunoblot analysis. Abcc2, Abcc3, and Abcb1 efflux drug transporters were significantly elevated in MCD livers (2.2-, 2.5-, and 2.4-fold, respectively); however, no significant alterations in Ugt1a1 protein levels were observed.

**EZE and EZE-GLUC Disposition in Diet-Induced NASH.** EZE is clinically administered as a 10-mg oral dose; however, because the intestinal expression of drug transporters important to the disposition of EZE has not yet been evaluated in the MCD diet rodent model of NASH, we chose to dose EZE both orally and intravenously. This allowed for separate determination of both the hepatic and intestinal contribution to EZE disposition in NASH. Figure 3 shows plasma concentrations of EZE, EZE-GLUC, and total EZE after either oral or intravenous dosing over a 120-min period. Total EZE and EZE-GLUC plasma concentrations were significantly elevated above control beginning at 40 min after oral dosing of EZE. This elevation above control persisted until conclusion of the experiment at 120 min. After intravenous dosing of EZE, MCD animals exhibited significantly higher plasma concentrations of total EZE throughout the study. Likewise, plasma concentrations of EZE-GLUC were significantly elevated in MCD animals beginning at 10 min and continuing throughout the study. No significant changes in the sinusoidal efflux of EZE were observed between diet groups regardless of dosing method.

Biliary concentrations of EZE, EZE-GLUC, and total EZE are shown in Fig. 4. Total EZE and EZE-GLUC concentrations in MCD rodent bile were decreased from control in orally dosed animals; however, these results were not significant. Likewise, EZE-GLUC biliary concentrations were consistently lower in intravenously dosed MCD animals, but not to a significant extent. EZE biliary concentrations in intravenously dosed MCD animals were significantly decreased from control beginning at 30 min and continuing until 90 min.

Urine samples were collected by bladder puncture at the conclusion of the 120-min experiment. Urinary concentrations of EZE, EZE-GLUC, and total EZE in control and MCD animals are shown in Fig. 5. Total EZE and EZE concentrations were significantly higher in orally dosed MCD animals than in control animals. Analysis of
intravenously dosed animals revealed a significant increase in EZE, EZE-GLUC, and total EZE urinary concentrations in MCD animals. Hepatic tissue concentrations of EZE, EZE-GLUC, and total EZE are shown in Fig. 6. No significant alterations in tissue retention of total EZE, EZE, or EZE-GLUC were observed for either orally or intravenously dosed animals.

Effect of Diet-Induced NASH on Bile Flow and Biliary Bile Acid Concentrations. Bile volume and flow rate throughout the 120-min experiment were calculated assuming a specific gravity of 1.0; results are shown in Fig. 7. Regardless of dosing, bile volume was unaltered in MCD animals. The bile flow rate, normalized to body weight, was elevated in MCD animals. Bile acid concentrations in bile samples of control and MCD rats were determined spectrophotometrically by the enzyme cycling method using a Diazyme Total Bile Acids Assay Kit. This analysis was performed to determine the functionality of bile acid excretion processes in the liver. Regardless of dosing method, total bile acid levels in bile were unaltered between diet groups.

Effect of Diet-Induced NASH on Intestinal Ugt1a1 Expression. mRNA expression of Ugt1a1 in control and MCD duodenum and ileum is shown in Fig. 8. This analysis was conducted to determine whether disruption of EZE metabolism in the gut of orally dosed animals underlies the effect of NASH on EZE disposition. However, no significant alterations in Ug1t1a1 transcriptional regulation were detected in rodent intestine. Immunoblots and densitometric results of relative Ug1t1a1 protein levels are shown in Fig. 8. Similar to Ug1t1a1 mRNA, Ug1t1a1 protein showed no significant alterations between control and MCD.

Efflux Drug Transporter Localization in Diet-Induced NASH. Immunohistochemical staining of Abcc2 and Abcb1 in control and MCD formalin-fixed, paraffin-embedded liver samples is shown in Fig. 9 at 40× magnification. Staining of both Abcc2 and Abcb1 in MCD livers appears to be pulling away from the canalicular membrane (Fig. 9, circled in red), suggesting that the transporters may be internalized in diet-induced NASH. Staining for both transporters appeared to be properly localized along the canalicular membrane in control livers.

Discussion
Despite the current prevalence and projected continuation in the growth of occurrences of NAFLD, little information is available concerning the expression or function of drug-metabolizing enzymes and transporters in patients with NAFLD. Recent studies in our laboratory have investigated drug-metabolizing enzymes and transporters in both human samples and rodent models. These studies have included the identification of perturbations in cytochrome P450...
(Fisher et al., 2009b) and glutathione transferase (Hardwick et al., 2010) enzyme expression and functionality in a population of human steatosis and NASH samples. In addition, alterations in the efflux of acetaminophen (Lickteig et al., 2007) and uptake of bromosulfophthalein (Fisher et al., 2009a) have been investigated in rodent models of NAFLD. However, information concerning the mechanisms behind these alterations in drug disposition and, more importantly, how they may manifest clinically in the human disease is lacking. Nonetheless, it can be hypothesized that NAFLD has significant potential to alter the absorption, distribution, metabolism, and elimination of several pharmaceutical agents. Because of the pervasiveness of NAFLD within the general population and the propensity for these patients to be medicated for multiple symptoms of the metabolic syndrome, knowledge of the effect of NAFLD on absorption, distribution, metabolism, and elimination could be valuable in identifying patients at risk for adverse drug reactions.

In the current study, we have demonstrated that the MCD diet rodent model of NASH causes a significant increase in the amount of the pharmacologically active metabolite EZETIMIBE excreted into sinusoidal blood and away from its target site. However, because of the inherent characteristics of the MCD model, NASH rodents presented with significantly lower total body weights before the initiation of disposition experiments. Because of the reduction in total body weight characteristic of the model, animals were dosed on the basis of body weight. On examination of the parent drug, EZETIMIBE, in the plasma of intravenously dosed animals, it appears that overall drug exposure is not significantly different between control and NASH rodents, leading us to conclude that the observed changes in body weight most likely have little effect on the observed alterations in drug disposition. In contrast, specific alterations in exposure to the metabolite, EZETIMIBE-GLUC, were observed in NASH rodents, and we have proposed a possible mechanism for this observation. In general, elevated plasma efflux of a drug results in increased systemic exposure to the drug in the form of retention within the systemic circulation. This could have profound effects on toxicity in extrahepatic tissues such as the kidney. However, for EZETIMIBE, increased efflux of EZETIMIBE-GLUC into sinusoidal blood, as seen in NASH rodents, and diminished efflux into bile would mean that less drug is being delivered to the site of action in the small intestine. This could have implications for EZETIMIBE...
efficacy and could possibly play a role in selection of the most appropriate therapeutic option for patients with NASH.

The plasma efflux of EZE-GLUC is similar to that of acetaminophen-glucuronide in MCD animals as demonstrated by Lickteig et al. (2007). In particular, acetaminophen-glucuronide excretion in bile was diminished in MCD animals, and plasma efflux was significantly increased. Evidence from Lickteig et al. (2007) and the current study suggests that changes in disposition of glucuronide metabolites of additional pharmaceutical agents is likely to occur in patients with NASH. The conclusion of Lickteig et al. (2007) that increased plasma efflux of drug in the MCD model could be due to the combination of increased expression and affinity for Abcc3 is certainly plausible in the current study. de Waart et al. (2009) conducted inhibition experiments in membrane vesicles containing either Abcc3 or Abcc2 and discovered that EZE-GLUC is able to inhibit the transport of estradiol-17β-glucuronide more efficiently for Abcc3 than for Abcc2. Furthermore, studies have shown that Abcc3 preferentially transports glucuronide metabolites, and there is evidence for higher affinity over that for Abcc2. Chu et al. (2004) have identified ethinylestradiol glucuronide as a higher affinity substrate for ABCC3 than ABCC2. Further evidence implicating Abcc3 as the major transporter of glucuronide conjugates stems from studies in Abcc3 knockout mice, which exhibit significant hepatic retention of acetaminophen-glucuronide compared with wild-type animals without alterations to Abcc2 protein levels (Manautou et al., 2005). Thus, the increased expression of Abcc3 in MCD rodents and preferential affinity for Abcc3 may help to explain, at least in part, the elevation in plasma efflux of EZE-GLUC.

Further evidence supporting the conclusion that NASH may cause a shift in the disposition profile of clinically relevant drugs is seen in the urinary excretion of total EZE and EZE.
Drug because of the hydrolysis of EZE-GLUC after excretion into bile (Kosoglu et al., 2005). MCD animals exhibited higher concentrations of both total EZE and EZE in the urine than controls. This finding is similar to results shown in Abcc2-deficient rats in which increased serum concentrations of EZE-GLUC resulted in increased renal excretion and decreased fecal excretion of EZE and EZE-GLUC (Oswald et al., 2006b), indicating that the shift in the hepatic elimination of EZE from bile to plasma can have a significant influence on drug efficacy.

It is interesting to note that in the current study, diminished biliary concentrations of total EZE and EZE-GLUC were observed in MCD animals. However, these results did not reach significance. Analysis of bile volume revealed no significant differences between control and MCD animals. However, the bile flow rate, which is a calculation of bile volume normalized to body weight, was elevated in MCD animals. This elevation in bile flow is reflective of the well documented reduction in body weight that occurs with the MCD diet (Fan et al., 2009; Schattenberg and Galle, 2010). In addition, bile acid concentrations were not altered. These data suggest that diminishment of biliary drug excretion is not simply due to cholestatic conditions and that, instead, a more specific mechanism may be responsible. Immunohistochemical staining of Abcc2 and Abcb1, both of which play a major role in the biliary excretion of EZE-GLUC and its repeated delivery to the site of action, revealed a unique mechanism of transport regulation. In the livers of MCD rodents, cellular localization of both Abcc2 and Abcb1 may be internalized away from the canalicular membrane, whereas localization of Abcg2 (an additional biliary transporter) remains unchanged (data not shown). Disrupted localization of specific transporters would make them unavailable for successful transport of drugs and their metabolites in bile, despite an induction of protein levels. Blocking of biliary transport by way of altered localization of efflux drug transporters could, in conjunction with increased expression of Abcc3 on the sinusoidal membrane, drive the shift from biliary to plasma efflux. Likewise, Mottino et al. (2002, 2005) have shown that altered localization of Abcc2 during estradiol-17β-glucuronide-induced cholestasis results in reduction of the biliary concentration of Abcc2 substrates. Zhang et al. (2005) observed a similar phenomenon in sandwich-cultured rat hepatocytes. The biliary excretion of 5-(6)-carboxy-2',7'-dichlorofluorescein was diminished when Abcc2 was internalized. Of particular importance in the current study is the possible internalization of not just one, but two, efflux drug transporters on the canalicular membrane. Although brightfield immunohistochemical staining suggests that localization of these transporters indeed appears to be disrupted in MCD livers, further investigation by confocal microscopy has been limited because of severe autofluorescence in the livers of MCD animals. However, several experiments have shown that internalization of Abcc2 alone can significantly disrupt biliary drug efflux and that altered cellular localization of both Abcc2 and Abcb1 could have confounding effects on biliary excretion in NASH. In addition, the level of bile acids observed in the bile of control and MCD rodents indicates that bile acid secretion is intact in this disease model. This finding supports the conclusion that the alterations we have discovered in EZE disposition are probably due to expression and localization changes in specific transporters responsible for EZE disposition rather than to a general cholestatic phenomenon.

An additional explanation for the observed results with biliary excretion of EZE in MCD animals arises from studies in knockout animals. de Waart et al. (2009) have revealed a very complex interplay of efflux transporters in the disposition of EZE. Biliary excretion of EZE in Abcg2 (Bcrp) knockout mice revealed no significant alterations compared with those in wild-type mice 2 h after dosing. As an alternative, Abcc2 knockout and Abcc2/Abcg2 double knockout mice exhibited a reduction in biliary excretion of EZE to 56 and 2.5% of controls, respectively (de Waart et al., 2009). This indicates that although Abcg2 is not a major transporter of EZE-GLUC under normal circumstances, it may be capable of partially compensating for the loss of Abcc2 and, as observed in the current study, for Abcb1 as well. Although expression of Abcg2 was not evaluated in the current study, Lickteig et al., 2007 found an elevation of Abcg2 protein in the MCD diet rodent model of NASH, thus lending support to our observations that the discrepancy in EZE biliary excretion in NASH may be due to multiple overlapping substrate specificities as well as to disruption in cellular localization of Abcc2 and Abcb1.

To further demonstrate the role of hepatic transporters and rule out a change in metabolism in the disposition of EZE, we examined expression of Utg1a1 in the intestine and liver. Potential alterations of metabolism in the gut of orally dosed animals could confound the
disposition results acquired in the study. Likewise, changes in hepatic metabolism of intravenously dosed animals would diminish our ability to determine the effect of the liver on alterations of disposition in NASH. However, no significant alterations in Ugt1a1 at the mRNA or protein level were observed in the liver or intestine of MCD animals, indicating that the alterations in EZE-GLUC plasma levels in MCD animals is not due to metabolism. In addition, previous studies in our laboratory identified a reduction in uptake transport function in MCD animals (Fisher et al., 2009a). We determined concentrations of EZE, EZE-GLUC, and total EZE in hepatic tissue of control and MCD animals to identify whether NASH affects uptake of EZE into hepatocytes. No significant changes in drug concentration within the hepatic tissue of MCD animals was observed, indicating that entry of drug into hepatocytes was not a confounding factor in the measured elevations of plasma drug concentrations in NASH.

In conclusion, the combination of altered cellular localization of the biliary efflux drug transporters Abcc2 and Abcb1 and the induction of the higher affinity sinusoidal efflux drug transporter, Abcc3, in rodent NASH drives a shift from primarily biliary efflux of EZE-GLUC to increased plasma concentrations and elevated urinary excretion. This plasma retention of drugs may have an implication for therapeutic efficacy and the potential risk of adverse drug reactions for many pharmaceuticals administered to patients with NASH.

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Authorship Contributions

Participated in research design: Hardwick, Fisher, and Cherrington. Conducted experiments: Hardwick, Fisher, Street, and Canet. Performed data analysis: Hardwick and Cherrington. Wrote or contributed to the writing of the manuscript: Hardwick and Cherrington.

References


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